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## Review

# Activation of the tumor suppressor p53 upon impairment of ribosome biogenesis<sup>☆</sup>

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## ABSTRACT

Errors in ribosome biogenesis can result in quantitative or qualitative defects in protein synthesis and consequently lead to improper execution of the genetic program and the development of specific diseases. Evidence has accumulated over the last decade suggesting that perturbation of ribosome biogenesis triggers a p53-activating checkpoint signaling pathway, often referred to as the ribosome biogenesis stress checkpoint pathway. Although it was originally suggested that p53 has a prominent role in preventing diseases by monitoring the fidelity of ribosome biogenesis, recent work has demonstrated that p53 activation upon impairment of ribosome biogenesis also mediates pathological manifestations in humans. Perturbations of ribosome biogenesis can trigger a p53-dependent checkpoint signaling pathway independent of DNA damage and the tumor suppressor ARF through inhibitory interactions of specific ribosomal components with the p53 negative regulator, Mdm2. Here we review the recent advances made toward understanding of this newly-recognized checkpoint signaling pathway, its role in health and disease, and discuss possible future directions in this exciting research field. This article is part of a Special Issue entitled: Role of the Nucleolus in Human Disease.

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## 1. Introduction

The mammalian ribosome is a complex structure composed of four RNAs (rRNAs) and 80 distinct proteins (RPs) [1,2]. Increased capacity for mRNA translation by way of ribosome biogenesis dictates the capacity of cells to grow, proliferate and differentiate [3–6]. Most steps in ribosome biogenesis are temporally and spatially organized within the nucleolus, where a 47S rRNA precursor is transcribed by RNA polymerase I, processed and modified by more than 150 non-ribosomal proteins and 100 small non-coding RNAs into the mature 18S rRNA of the 40S ribosomal subunit and to 5.8S and 28S rRNAs of the 60S ribosomal subunit [7–9]. Although the genes for 5S rRNA are transcribed in the nucleus by RNA polymerase III, 5S rRNA is assembled into the 60S ribosomal subunit in the nucleolus [8,10]. The RP mRNAs are transcribed in the nucleus by RNA polymerase II and translated on cytoplasmic ribosomes, imported into the nucleus, and assembled with the rRNA while the rRNA is processed in the nucleolus [11–15]. Specific RPs join nascent 60S and 40S subunits during their path from the nucleolus to the cytoplasm [11,14,15]. In addition to their roles in stabilization and promotion of correct folding of rRNAs for ribosome assembly, RPs are involved in export of ribosomal precursors and regulation of specific

steps in protein synthesis [11,14,15]. However, the exact requirement of individual RPs for different stages of ribosome biogenesis and/or distinct steps of mRNA translation process in mammals is just beginning to be understood [11,14,15].

Given the enormous energy investment in ribosome biogenesis, the proper execution of this component of the genetic program has high importance. Errors in this process can result in the development of a number of pathological conditions. We and others have hypothesized that molecular mechanisms must have evolved to sense the fidelity of this critical cellular process to prevent the development of disease [6,16,17]. The first indication of this came from a study using an inducible model for deleting the gene encoding the S6 ribosomal protein, *Rps6*, in the liver of adult mice, which led to abrogation of nascent 40S ribosome biogenesis and abolition of cell proliferation in the liver following partial hepatectomy [17]. These results could not be simply attributed to the loss of protein synthetic capacity in these cells as pre-existing ribosomes provided sufficient translational capacity to increase liver size in response to a fasting/re-feeding regimen, a process that primarily involves an increase in cell mass but not in cell number. These observations suggested the existence of a novel cell cycle checkpoint triggered by impaired ribosome biogenesis [17], in a manner analogous to of checkpoints triggered by DNA damage [18]. Studies over the last decade have convincingly demonstrated that perturbation of ribosome biogenesis activates the tumor suppressor p53 *via* binding of several ribosomal components to its negative regulator, Mdm2, independent of DNA damage [19–22]. The p53 is best known for its role as

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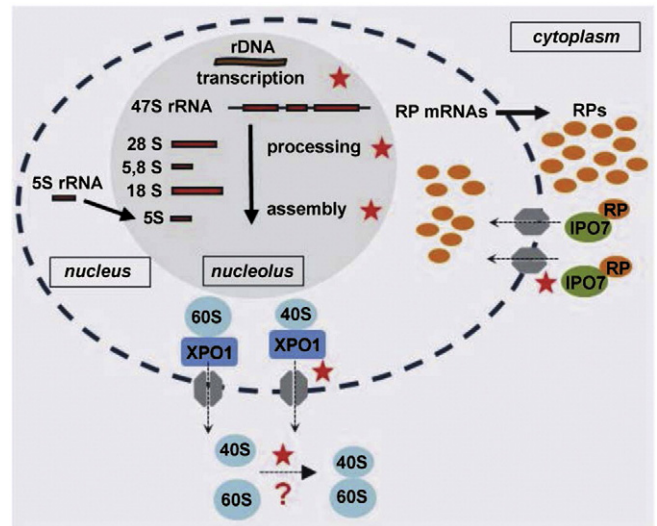
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a tumor suppressor. The exposure of cells to various stressors activates p53, which regulates transcription of many coding and noncoding genes, with ensuing multiple outcomes including cell cycle arrest, senescence, apoptosis, changes in metabolism, and DNA repair [23,24]. Disruption of these functions allows continuous proliferation, genomic instability and evolution of stress-damaged cells, resulting in their illegitimate survival and malignancy [24,25]. Given that the loss of wild-type p53 provides many selective advantages to cells, it comes as no surprise that half of all human malignant tumors have mutations within the *TP53* gene [26]. In cancers retaining wild-type p53, the functions of p53 are likely inactivated by defects in upstream or downstream p53 network components [23]. As an abundance of evidence has shown, inherited and acquired abnormalities in ribosome function can lead to tumorigenesis [27,28] and thus it can be speculated that induction of a p53-dependent checkpoint response might prevent expansion of such potentially hazardous cells [29]. Although there are some indications in support of this idea, definitive evidence has not yet been provided [30]. Recent evidence has shown that p53 activation upon impairment of ribosome biogenesis can also be responsible for certain pathological manifestations in mice and humans [31–35].

## 2. Impairment of ribosome biogenesis at various stages can activate the p53 tumor suppressor

### 2.1. Evidence from cell culture studies

Following the study of mice with liver-specific inducible deletion of the *Rps6* gene that demonstrated the existence of a previously unrecognized checkpoint triggered as a result of deficiency in ribosome biogenesis [17], Pestov and co-workers provided the first evidence that the p53 tumor suppressor is a component of this pathway [20]. They demonstrated that the expression of dominant negative mutants of Bop1, which inhibit rRNA processing, prevented cell cycle progression in a p53-dependent manner. A number of subsequent investigations have demonstrated that other perturbations of ribosome biogenesis in cell culture can also trigger the p53 response [22]. That the inhibition of rRNA transcription can lead to functional alterations of the nucleolus and upregulation of p53 protein has been demonstrated in many different ways including: genetic inactivation of the RNA polymerase I (Pol I) transcription factor TIF-1A [36], blockage of Pol I transcription factor UBF by microinjection of specific monoclonal antibodies [37], silencing the *POLR1A* gene coding for the Pol I catalytic subunit [38], treatment with the immunosuppressant mycophenolic acid [39,40], low concentrations (<10 nM) of actinomycin D, which intercalates into the GC-rich regions of rDNA [37,41] or the small molecule compound CX-3543 (quarfloxin) that impairs binding of SL1/TIF-1B to the rDNA promoter leads to functional and morphological alterations of the nucleolus and stabilization of p53 protein levels [42]. Furthermore, the inhibition of rRNA processing by treatment with a chemotherapeutic compound 5-fluorouracil [43,44] or decreased expression of proteins required for maturation of 18S and 28S rRNA such as hUTP18 [45], PAK1IP1 [46], WDR3 [47], WDR12 [45], WDR36 [48], nucleophosmin (NPM, B23) [49], nucleostemin [50] as well as specific RPs of either 40S or 60S [51] including RPS6 [51], RPS9 [52], RPL23 [53–55], RPL7a [51], RPS7 [51,53,54], RPL24 [31], RPL26 [53], RPL29 [56], RPL30 [56], RPL37 [57], RPS14 [58,59], RPS19 [58], RPS15, RPS20 and RPL37 [60] can also induce a p53-mediated stress signal. Additionally, it has been recently demonstrated that the inhibition of RP nuclear import or nuclear export of ribosomal subunits by depletion of importin 7 (IPO7) or exportin 1 (XPO1), respectively, perturbs ribosome biogenesis, and consequently triggers the p53 response [61]. Taken together, inhibition of ribosome biogenesis at various levels consistently leads to p53 accumulation (Fig. 1). However, it remains to be determined how various lesions in ribosome biogenesis are sensed by this p53-dependent checkpoint mechanism.



**Fig. 1.** Impairment of ribosome biogenesis at various steps can trigger p53 upregulation. Inhibition of Pol I transcription, rRNA processing, assembly of ribosomal subunits, RP nuclear import by depletion of importin 7 (IPO7) or nuclear export of 40S and 60S ribosomal subunits by the depletion of exportin 1 (XPO1) can lead to p53 upregulation (indicated by red stars). p53 upregulation by impaired association of the 40S and 60S subunits has not been unambiguously demonstrated (question mark).

### 2.2. Evidence from in vivo studies

The first *in vivo* evidence implicating p53 as the critical checkpoint component triggered by deficiency in ribosome biogenesis was obtained by studies of mouse lines employing T cell-specific [21] and ubiquitous deletions of one *Rps6* allele [34]. Conditional deletion of one *Rps6* allele in T cells inhibited their accumulation in the spleen and lymph nodes, because of decreased survival. Additionally, T cell receptor-mediated stimulation of *Rps6*-heterozygous T cells induced a normal increase in their size, but cell cycle progression was impaired. Genetic inactivation of p53 in *Rps6*-haploinsufficient T cells rescued this proliferative defect and restored normal numbers of T lymphocytes in the peripheral lymphoid organs, suggesting that the defect in ribosome biogenesis activated a p53-dependent apoptosis and cell cycle checkpoints to prevent the survival and proliferation of defective T lymphocytes [21]. To investigate the response to *Rps6*-deficiency in the whole organism, one *Rps6* allele was conditionally deleted in growing mouse oocytes and *Rps6*-heterozygous embryos generated [34]. Embryonic development up to embryonic day 5.5 (E5.5) was unaffected. However, gastrulating *Rps6*-heterozygous embryos (E5.5–E7.5) displayed a dramatic increase in p53 protein levels, inhibition of cell cycle progression and apoptosis, which resulted in embryonic lethality at this developmental period, at which under normal conditions ribosome biogenesis and cell proliferation are dramatically upregulated. Inactivation of p53 in *Rps6*-heterozygous embryos bypassed this gastrulation checkpoint and allowed development until E12.5, when they died with diminished fetal liver erythropoiesis and severe placental defects, most likely because a defective translation of specific mRNAs or an uncharacterized p53-independent checkpoint response [34].

In contrast to *Rps6*-heterozygous mice, *Rpl22*-heterozygous mice showed no obvious pathological phenotype. However, *Rpl22*-null mice displayed a selective defect in the development of  $\alpha\beta$ -lineage but not  $\gamma\delta$  T cells, which was rescued in a p53-negative genetic background [62]. More recently, the role of p53 in the phenotype of the Belly Spot and Tail (*Bst*) mouse that carries a hypomorphic mutation in one allele of the *Rpl24* gene has been analyzed [31]. *Rpl24*<sup>Bst/+</sup> mice reach adulthood and display a number of pathological phenotypes including reduced body size, a white ventral middle spot, retinal abnormalities, a kinked tail, and other skeletal abnormalities. It was demonstrated

that, similar to *Rps6*-heterozygous embryos, p53 protein was aberrantly expressed in gastrulating *Rpl24<sup>Bst/+</sup>* embryos. However, the majority of the animals survive, presumably because levels of p53 protein in these embryos at E6.5 were significantly lower than in *Rps6*-heterozygote embryos and thus insufficient to trigger apoptosis and inhibit cell cycle progression [31,34]. Because the *Bst* mutation is a hypomorphic heterozygous mutation, another possibility exists that the expression level of *Rpl24* was above the required threshold for triggering the p53 checkpoint. Interestingly, p53 is aberrantly expressed in highly proliferative cells in the neural tube of *Rpl24<sup>Bst/+</sup>* embryos at midgestation. Genetic inactivation of p53 in these mice largely rescued all pathological phenotypes, suggesting that they are caused by p53. However, in the absence of p53 a large majority of *Rpl24<sup>Bst/+</sup>* mice die within two days of birth, suggesting a novel role for this tumor suppressor in organismal survival [31]. The study of *Rpl27a*-heterozygous mice further illustrated the high phenotypic diversity of RP-deficient mice [63]. These mice display epidermal hyperpigmentation, low body weight and anemia. Additionally, they exhibit abnormally low proliferation and high apoptosis levels in the cerebellar granule cell layer, which is probably the cause of ataxia in these mice. Similar to the above-mentioned RP-deficient mice, most of pathological phenotypes were rescued in a p53 deficient genetic background [63]. These studies strongly suggested that p53 activation upon impairment of ribosome biogenesis in RP-deficient mice is responsible for their specific pathological manifestations. However, it will be a huge challenge to identify p53-dependent biological responses that are triggered by various RP-deficiencies *in vivo* and determine their role in such diverse pathological phenotypes.

### 3. Molecular mechanisms of p53 activation

#### 3.1. p53 activation by DNA damage or oncogenes

The major cellular stressors that lead to cancer trigger activation of the DNA damage response (DDR) and the CDKN2A alternative reading frame (ARF) signaling pathways, which act as anti-cancer barriers by activating the tumor suppressor p53 [18,23,64]. Growing evidence over the past decade has demonstrated that perturbation of various steps of ribosome biogenesis can activate the p53 tumor suppressor independent of DNA damage and the ARF tumor suppressor via binding of several RPs to p53 negative regulator, Mdm2. To better understand this novel signal transduction pathway, we will briefly describe what is known regarding p53 activation by DNA damage and oncogenic activation. In unstressed cells, p53 protein levels and its transcriptional activity are negatively regulated by Mdm2, a RING finger E3 ubiquitin ligase [65,66]. Mdm2 becomes an active E3 upon heterodimerization with its homolog MdmX, which does not have E3 ligase activity itself [67–71]. This heterodimer promotes nuclear export of p53 and its proteasomal degradation via monoubiquitination or polyubiquitination, respectively, as well as inhibiting Mdm2 auto-ubiquitination and degradation [72]. Furthermore, both Mdm2 and MdmX directly repress p53 transcriptional activity [66,68]. The DDR signaling pathway leads to post-translational modifications of both p53 and Mdm2–MdmX, which disrupts their interaction and increases Mdm2–MdmX auto-degradation, leading to p53 stabilization and transcriptional activation [23,73–77]. Oncogenes, such as Ras, c-Myc, E2F, E1A,  $\beta$ -catenin, and v-abl, accelerate cell cycle progression, inducing stalling and collapse of DNA replication forks, which in turn leads to formation of DNA double-stranded breaks (DSB) and p53 activation via the DDR signaling as described above [78]. Additionally, oncogenes increase the binding of ARF to Mdm2's central acidic domain, which inhibits p53 ubiquitination and promotes MdmX degradation, thereby activating p53 [64,79–89]. Because Mdm2 is a transcriptional target of p53, an autoregulatory feedback loop is generated, which ensures that p53 is downregulated once the stressor or its consequences are removed [66].

#### 3.2. Regulation of p53 activation upon impairment of ribosome biogenesis: the central role of RPL5 and RPL11

The first suggestion that RPs may play a role in p53 activation came from the observation that RPL5 forms an extraribosomal complex with Mdm2, p53 and 5S rRNA [90]. Surprisingly, this finding did not attract much attention at that time. However, a decade ago, a few landmark studies implicated RPL5 and RPL11 as Mdm2 binding partners that block the E3 function of Mdm2 to promote p53 accumulation [91–93]. The fact that the depletion of either RPL5 or RPL11 is sufficient to suppress p53 upregulation upon inhibition of ribosome biogenesis at various levels suggests that RPL5 and RPL11, in a mutually dependent manner, are essential for this response [19,22,53,54,94,95]. The interaction between RPL5 and RPL11 with Mdm2 does not seem to be a unique phenomenon since it was shown that overexpressed RPL23 [55,93,96], RPL26 [97,98], RPS3 [99], RPS7 [100,101], RPS14 [59], RPS25 [102], RPS27A [103], RPS27, RPS27L [104], RPS15, RPS20 and RPL37 [60] can bind to Mdm2 and inhibit the ubiquitin protein ligase function toward p53, leading to p53 upregulation.

Given that RPs are among the most abundant proteins in mammalian cells and are highly basic, and thus can specifically or non-specifically interact with other proteins, experiments in which overexpressed RPs were used to demonstrate their interaction with the acidic domain of Mdm2 must be interpreted with caution [105]. Similarly, non-specific interactions between ribosomes or abundant free RPs with Mdm2 may occur in total cell lysates, where ribosomes can be partially disintegrated. Indeed, a recent study showed that all tested RPs coimmunoprecipitated with anti-Mdm2 antibodies from the total cell lysate, including RPL5, RPL11, RPL23, RPL26 and RPS6 [53].

The observation that the depletion of either RPL5 or RPL11 abolishes p53 upregulation in response to inhibition of rRNA transcription, rRNA processing, import of RPs, and export of immature ribosomal subunits, raises the question of the role of other Mdm2-binding RPs, which in the absence of RPL5 and RPL11, would have been predicted to still bind and inhibit Mdm2, upregulating levels of p53 [19,22,94]. The depletion of either RPL5 or RPL11 inhibits ribosome biogenesis [54,106]. However, this doesn't trigger the p53 response, probably because RPL5 and RPL11 are essential transducers of p53-activating signals upon ribosome biogenesis stress [19,22,53,54,94]. In contrast, inhibition of ribosome biogenesis caused by the depletion of RPL23, RPL26 and RPS7, like the depletion of other RPs, except for RPL11 and RPL5, activates p53 in an RPL5- and RPL11-dependent manner [53,54]. Previously published studies showed that the depletion of RPS7 [100,101], RPL23 [55,96] or RPL26 [98] also compromises the induction of p53 after pharmacological inhibition of ribosome biogenesis. Consistent with the fact that the depletion of specific RPs can decrease the number of ribosomes, a recent study showed that the inhibitory effects of RPS7 and RPL23 depletion on p53 accumulation induced by actinomycin D may be attributed to effects on global translation, rather than the loss of their specific effects on the Mdm2 function [54]. In fact, the depletion of RPs that do not bind to Mdm2 (i.e. RPS6 and RPL7a) exerts the same effect on actinomycin D-induced p53 accumulation [54].

Taken together, these observations highlight the centrality of RPL5 and RPL11 for p53 up-regulation in response to various ribosomal stressors. Consistent with this, RPL5 and RPL11, but not RPL23, RPL26, RPS3 and RPS7, accumulated in nonribosomal fractions upon various impairments of ribosome biogenesis, where they interacted with Mdm2 [53]. In addition to a number of cell culture studies, the RPL5–RPL11–Mdm2–p53 pathway has been convincingly supported by an *in vivo* mouse model. Knock-in mice that express a mutant Mdm2 (Mdm2<sup>C305F</sup>) that cannot bind RPL5 and RPL11 retain normal p53 response to DNA damage, but have impaired p53 activation upon perturbations in ribosome biogenesis [30,94]. The differences between these studies could also be due to time of treatment with pharmacological inhibitors of ribosome biogenesis, siRNA sequences used, or cell-line differences. Given that these early studies have convincingly shown that



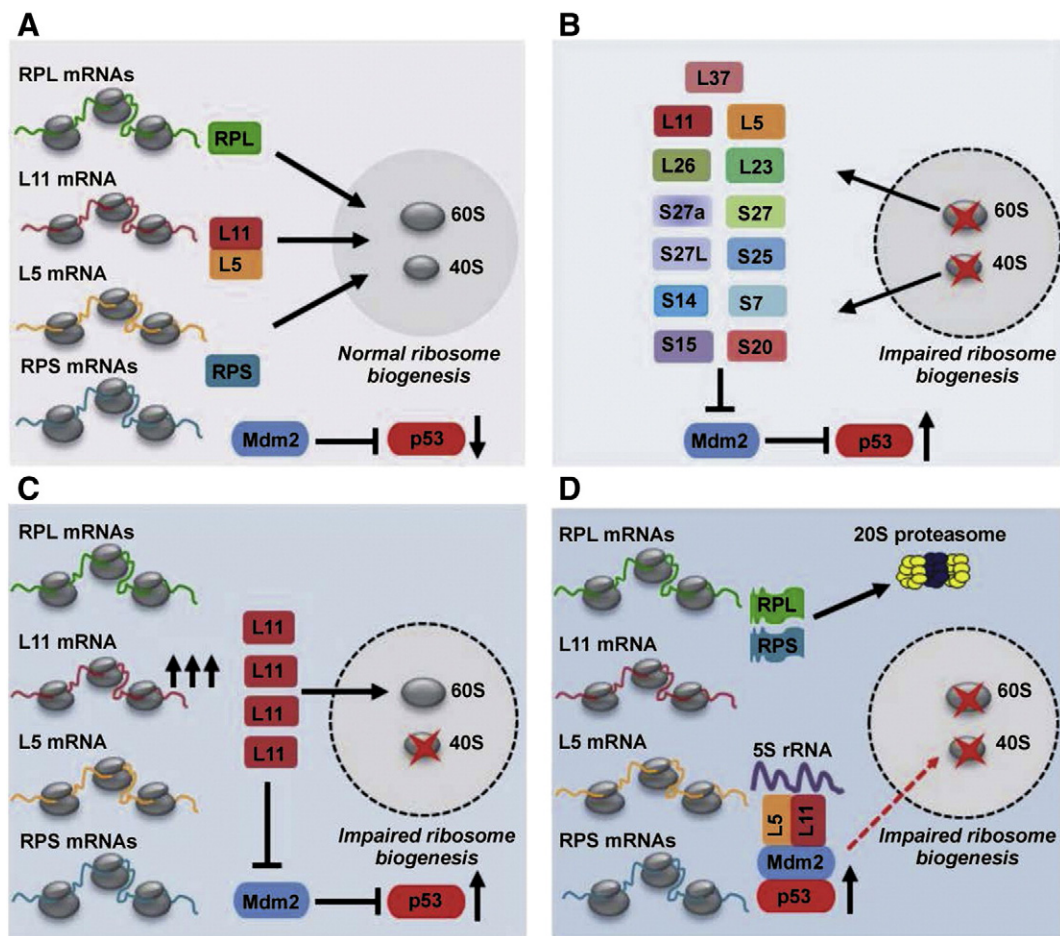
RPS7 [100,101], RPL23 [55,96] and RPL26 [98] interact with Mdm2 and efficiently inhibit its ubiquitin protein ligase activity toward p53, there is the possibility that they can regulate p53 levels in certain cell types or upon specific impairments of ribosome biogenesis, or with kinetics different from RPL5 and RPL11. Additionally, as previously shown for nucleolar factor nucleostemin [50], RPS7, RPL23 and RPL26 and possibly some other RPs may also trigger p53 activation when they are made in excess independent of “ribosome biogenesis stress,” which may occur in certain situations, as, for example, when c-Myc becomes overexpressed [61].

Given technical difficulties in studying signaling functions of RPs as mentioned above, it will be important to carefully design and interpret the experiments before unambiguously ascribing a “p53-activating” function to some of the previously suggested RPs or future candidate RPs.

### 3.3. The source of ribosome-free RPL5 and RPL11 upon ribosome biogenesis stress

Considerable controversy exists regarding the source of ribosome stress-induced ribosome-free “p53-activating” RPs. During cell growth,

RPs are assembled into nascent ribosomes (Fig. 2A). Earlier work suggested that perturbation of ribosome biogenesis causes nucleolar disruption and passive diffusion of a number of RPs, including RPL5, RPL11, RPL23, RPL26, and RPS7, from the nucleolus to the nucleoplasm, where they bind to Mdm2 and inhibit its ubiquitin protein ligase function toward p53, leading to p53 upregulation [19,22,94] (Fig. 2B). Alternatively, one study proposed that upon overexpression of RPL11, Mdm2 enters the intact nucleolus to interact with unassembled RPL11 [91]. In fact, others have shown that nucleolar disruption is not necessary to activate p53 upon alteration of ribosome biogenesis [38,51]. Production and nuclear import of RPs exceeding the needs of ribosome biogenesis could be the only condition required to activate the p53 [38]. Indeed, when 40S ribosome biogenesis is impaired, p53 is upregulated in an RPL11-dependent manner, in the absence of any significant effect on 60S ribosome biogenesis and nucleolar integrity [51] (Fig. 2C). Under these conditions, the cell selectively upregulates the translation of a group of mRNAs containing a 5' terminal oligopyrimidine tract (5' TOP) in the leader sequence, including the RPL11 mRNA [51,107]. The overproduced RPL11 presumably translocates to the nucleus where it interacts with and blocks the function of Mdm2, leading to p53 stabilization (Fig. 2C). However, the molecular mechanism by which the



**Fig. 2.** Three models explaining how p53 is upregulated upon ribosome biogenesis stress. (A) Under normal conditions, newly synthesized RPs of 40S (RPS) and 60S (RPL) ribosomal subunits are imported into the nucleolus, where they assemble with rRNA. Mdm2 is free to degrade p53. (B) Conditions that inhibit ribosome biogenesis cause nucleolar disruption and passive diffusion of several RPs from the nucleolus to the nucleoplasm, where they interact with Mdm2 and inhibit its ubiquitin protein ligase activity toward p53, leading to p53 upregulation. (C) Upon specific impairment of 40S biogenesis the cell selectively upregulates the translation of RP mRNAs, including the RPL11 mRNA. RPL11 presumably translocates to the nucleus and interacts with Mdm2, leading to p53 upregulation. In contrast, inhibition of 60S ribosome biogenesis impairs RPL11 mRNA translation. Under these conditions there are probably sufficient levels of free RPL11 to bind to Mdm2 and inhibit the ubiquitin protein ligase function toward p53. (D) Upon impairment of ribosome biogenesis, the majority of RPL and RPS are normally synthesized but are degraded by nuclear 20S proteasomes. In contrast, nascent RPL5 and RPL11 are mutually protected from degradation, and they accumulate outside of the ribosomes in a complex with 5S rRNA. This complex functions to inhibit Mdm2-mediated p53 degradation. Additional stress-induced signals might also increase the stability of the RPL5–RPL11–5S rRNA complex. A portion of the newly synthesized RPL5 and RPL11 continues to accumulate in nucleoli together with Mdm2 and p53 even after inhibition of ribosome biogenesis, suggesting that the altered nucleoli may provide a site for RPL5- and RPL11-dependent p53 activation. Less efficient import of nascent RPL5, RPL11 and 5S rRNA into the nucleolus upon inhibition of ribosome biogenesis may also contribute to their accumulation outside of the ribosomes (indicated by red dashed arrow).

lesion in ribosome biogenesis modulates translation of RP mRNAs has to be determined. In contrast, inhibition of 60S ribosome biogenesis impairs RPL11 mRNA translation [51,107]. Under these conditions there are probably sufficient levels of free RPL11 to inhibit Mdm2 and stabilize p53. Interestingly, upon concomitant inhibition of 40S and 60S ribosome biogenesis, induction of p53 is mediated by both of these mechanisms, resulting in supra-induction of p53 [54].

A new model envisages that upon inhibition of ribosome biogenesis, the majority of RPs are normally synthesized but are degraded by the ubiquitin-independent proteasomal degradation to prevent potentially toxic accumulation of unbound, free RPs in the cell [53,105,108–110] (Fig. 2D). In contrast, nascent RPL5, RPL11 and 5S rRNA are redirected from 60S ribosome biogenesis to Mdm2 inhibition in the cytoplasm and the nucleoplasm upon disruption of ribosome biogenesis [53,106]. These three components of the ribosome have been shown to depend on each other for Mdm2 binding and p53 induction [53,54,106]. It is possible that the mutual protection from the ubiquitin-independent proteasomal degradation and selective accumulation of ribosome-free RPL5 and RPL11 upon impairment of ribosome biogenesis [53] depends on the formation of the RPL5–RPL11–5S rRNA complex, which is under such condition redirected from ribosome biogenesis to Mdm2 inhibition [106] (Fig. 2D). Interestingly, the binding of ribosome-free RPL5 and RPL11 to Mdm2 is not required for their accumulation under these conditions [53,106]. The stability of the RPL5–RPL11–5S rRNA complex may also be increased *via* their post-translational modifications or binding to other factors under these conditions. However, no experimental evidence exists in support of this prediction.

An important challenge for future investigation is to rigorously validate these models and uncover novel components of this checkpoint signaling pathway and elucidate the molecular mechanisms of their regulation.

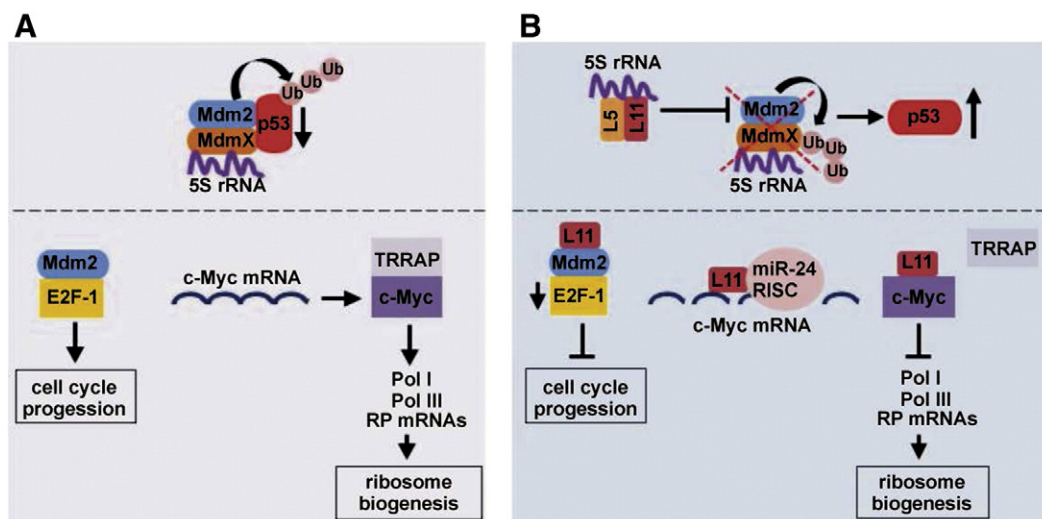
#### 3.4. Increasing complexity of the RP–Mdm2–p53 signaling pathway

A few new regulators of the RP–Mdm2–p53 signaling pathway have recently emerged. Under normal conditions, the Mdm2–MdmX complex represses the transcriptional activity of p53 and promotes its polyubiquitination [70] (Fig. 3A). Interestingly, 5S rRNA bound to MdmX in unstressed cells protects it from Mdm2-dependent ubiquitination and degradation, contributing to p53 inhibition [111]

(Fig. 3A). As mentioned in Section 3.3., upon the impairment of ribosome biogenesis, the RPL5–RPL11–5S rRNA pre-ribosomal complex is redirected from assembly into nascent 60S ribosomes to Mdm2 binding and inhibition of its E3 activity toward p53 [53,106,112]. Increased binding of RPL11 to Mdm2, in addition to inhibiting Mdm2-mediated degradation of p53, facilitates Mdm2-dependent MdmX ubiquitination and degradation, thus allowing full activation of p53 [111,113] (Fig. 3B). These results suggest that 5S rRNA may act as a positive or negative regulator of p53 depending on its association with the RPL5–RPL11–Mdm2 complex or MdmX, respectively. A recent study showed that RPS15, RPS20 and RPL37, in addition to inhibiting Mdm2-mediated degradation of p53, can downregulate MdmX levels but *via* distinct molecular mechanisms. RPS15 and RPS20 directly interact with MdmX and downregulate its protein levels, whereas RPL37 downregulates the MdmX mRNA levels [60]. Taken together, these results add to the complexity of the RP–Mdm2–MdmX–p53 network.

In yeast, two assembly factors, Rpf2 and Rrs1, are essential for assembling the RPL5–RPL11–5S rRNA complex into pre-ribosomes [114]. It was recently shown that their human homologues, hRrs1 and Bxdc1, are not essential components of the ribosome-free RPL5–RPL11–5S rRNA complex which regulates Mdm2 upon inhibition of ribosome biogenesis in human cells, suggesting that they lie at, or downstream of, the regulatory branch point that mediates the targeting of the RPL5–RPL11–5S rRNA precursor complex to inhibition of Mdm2 [106].

A new study suggests that PICT1 (protein interacting with the C terminus 1 of the tumor suppressor PTEN), also known as GLTSCR2 (glioma tumor suppressor candidate region 2 gene), is a negative regulator of RPL11-mediated p53 activation [115]. It was originally proposed that PICT1 is a tumor suppressor because it stabilizes phosphatase and tensin homolog (PTEN) [116] and its low expression in diffuse glioma and ovarian cancer is correlated with a more aggressive phenotype [116–118]. However, more recent studies suggested that PICT1 may not be a tumor suppressor. Patients with oligodendrogliomas with PICT1-haploinsufficiency have a better prognosis than other oligodendroglioma patients [119–121]. Similarly, colon and esophageal cancer patients whose tumors retain wild-type p53 lower expression of PICT1 appear to have a better prognosis [115]. Also, *PICT1*-heterozygous mice are more resistant to develop a chemically-induced papillomas compared to wild-type mice [115]. Consistent with these effects, the



**Fig. 3.** Mechanisms by which RPs regulate p53-dependent and -independent signaling pathways upon ribosome biogenesis dysfunction. (A) Under normal conditions, the Mdm2–MdmX–5S rRNA complex represses the transcriptional activity of p53 as well as polyubiquitinates p53. c-Myc promotes cell growth by upregulating ribosome biogenesis, whereas Mdm2 stabilizes E2F-1 to promote cell cycle progression. (B) Upon inhibition of ribosome biogenesis, the RPL5–RPL11–5S rRNA complex binds to the Mdm2–MdmX–5S rRNA complex, leading to its auto-degradation and release of p53. Furthermore, ribosome-free RPL11 binds to Mdm2 and inactivates its E2F-1 stabilizing function, leading to a p53-independent cell cycle arrest. RPL11 also binds c-Myc mRNA and there recruits miR-24/RISC to reduce the level of c-Myc mRNA. Moreover, ribosome-free RPL11 binds to c-Myc and blocks the recruitment of its transcriptional co-activator TRRAP to Pol I, Pol III and RP genes to inhibit ribosome biogenesis and cell growth.

depletion of PICT1 leads to induction of p53 and apoptosis. Mechanistically, PICT1 binds to and retains RPL11 in the nucleolus and its deletion allows the release of RPL11 to the nucleoplasm, where it binds to Mdm2 and blocks Mdm2-mediated ubiquitination [115]. Interestingly, it was suggested that RPL11 acts in an RPL5-independent manner under these conditions [115], contradicting the model in which RPL5 and RPL11 are mutually dependent in p53 activation [53,54,112,115].

NEDDylation is the process by which the ubiquitin like protein NEDD8 is conjugated to its target proteins. Recently, NEDDylation of RPL11 has been implicated in p53 stabilization and its transcriptional activation in response to inhibition of ribosome biogenesis by actinomycin D. In the absence of ribosome biogenesis stress, NEDDylation of RPL11 protects it from degradation and is required for its localization in the nucleolus [122]. However, actinomycin D treatment triggers a rapid RPL11 de-NEDDylation, thus causing its re-localization to the nucleoplasm, where it binds Mdm2. It was also suggested that Mdm2-mediated NEDDylation protects RPL11 from degradation in the nucleus, allowing Mdm2-mediated p53 stabilization upon actinomycin D treatment [122]. These findings are in contrast with two recent studies that showed accumulation of ribosome-free RPL11 upon actinomycin D treatment occurred even in the absence of Mdm2 and p53 [53,106]. This discrepancy certainly requires further investigation.

It is known that impairment of ribosome biogenesis by low dose actinomycin D treatment triggers p300/CBP-mediated p53 acetylation, which plays a role in the regulation of p53 stability and enhancement of p53-mediated transcription [123]. Mahata and coworkers showed that upon actinomycin D treatment NEDDylated RPL11 is rapidly but transiently recruited to promoter sites of p53 target genes in an Mdm2-dependent manner [124]. The binding of NEDDylated RPL11 relieves p53 from Mdm2-mediated transcriptional repression, suggesting yet another important level of p53 regulation by RPL11 [124].

Very recently, additional insights into regulation of p53 transcriptional activity in response to inhibition of ribosome biogenesis were obtained. Myb-binding protein 1a (MYBBP1A), which is localized in the nucleolus in an RNA-dependent manner, translocates to the nucleoplasm upon impairment of ribosome biogenesis [125]. This translocation is dependent on RPL5 and RPL11, and increase the interaction between p53 and p300/CBP to enhance p53 acetylation. Additionally, the promyelocytic leukemia (PML) tumor suppressor was implicated in regulating p53 acetylation on residue K382 upon low-dose actinomycin D treatment [123,126]. It was reported that PML co-localizes with p53, acetylated-p53 and Mdm2 in the disrupted nucleoli upon actinomycin D treatment in RPL5- and RPL11-dependent manner [53], implying a role for the nucleolus in p53 acetylation by ribosome biogenesis stress.

In conclusion, understanding the molecular mechanisms by which ribosome biogenesis stress regulates p53 acetylation and its transcriptional activation, as well as assessing the biological relevance of post-translational modifications require further investigation.

#### 4. p53-Independent signaling pathways downstream of RP-Mdm2 checkpoint activation by ribosome biogenesis dysfunction

Several studies reported the existence of p53-independent response pathways to impaired ribosome biogenesis [127]. Here we will focus on those involving common players of the canonical RP-Mdm2 pathway. Since growing evidence argues for p53-independent effects of Mdm2 in various biological processes including cell cycle control, apoptosis, differentiation, epithelial-to-mesenchymal transition, DNA repair, transcription, and other processes, the possibility exists that RPL5 and RPL11 may also influence these Mdm2 functions upon inhibition of ribosome biogenesis [128,129]. It has been demonstrated that impairment of rRNA transcription in cells with inactivated p53 results in inhibition of cell cycle progression via down-regulation of the transcription factor E2F-1. Mechanistically, RPL11 interacted with Mdm2 and inactivated

its E2F-1-stabilizing function under these conditions [130] (Fig. 3A and B).

It is possible that RPL11 may, in addition to Mdm2, bind to other partners upon ribosome biogenesis stress, thus expanding the repertoire of cellular biological processes it regulates. Under normal conditions c-Myc promotes cell growth by upregulating ribosome biogenesis (Fig. 3A) [4,131,132]. It was shown recently that upon inhibition of ribosome biogenesis RPL11 binds c-Myc mRNA at its 3' untranslated region (3'-UTR) and recruits micro-RNA-induced silencing complex (miRISC) and miR-24, leading to c-Myc mRNA degradation [133] (Fig. 3B). Under the same conditions, RPL11 also binds to the N-terminal transcriptional activation domain of c-Myc and inhibits the recruitment of its transcriptional coactivator, transformation/transcription domain-associated protein (TRRAP), at the promoters of its target genes driven by Pol I, Pol II or Pol III [61,134,135]. By down-regulating c-Myc expression and transactivation activity RPL11 inhibits ribosome biogenesis and cell growth upon ribosome biogenesis stress (Fig. 3B). A systematic approach for identifying direct and indirect RPL5 and RPL11 protein and RNA partners will be required to explore the full complexity of this novel and important signaling pathway. Potential linkages of RPL5 and RPL11 with characterized proteins and RNAs may suggest novel biological functions of RPL5 and RPL11 in response to defects in ribosome biogenesis.

#### 5. Quality control of ribosome biogenesis and degradation of defective ribosomes

The mechanisms by which various defects in ribosome biogenesis trigger p53-dependent and -independent biological responses, which probably allow adaptation of the cell to ribosome biogenesis stress, have been intensively studied in mammalian cells [22]. However, little is known about quality control mechanisms that prevent defective ribosomes from accumulating and generating translational errors and competing with functional ribosomes for amino acids, translation factors, and mRNAs [136,137]. A failure to properly recognize and degrade such defective ribosomes could also have deleterious consequences beyond the mRNA translation. For example, unassembled rRNA precursors could inappropriately interact with rDNA, leading to the formation of rRNA-rDNA hybrids, which may be a source of DSB and genomic instability [138,139], as well as alter normal functions of the nucleolus in various biological processes [140]. Upon impairment of ribosome biogenesis, it appears that there might be nuclear quality control processes in place to monitor aberrant pre-ribosomal assembly. Such defective ribosome intermediates can be prevented from exiting the nucleolus [9,141], where they are degraded by a process involving the Trf4/5, Air1, Mtr4 polyadenylation (TRAMP) complex. The TRAMP complex polyadenylates defective or naked rRNA substrates in nucleolar structure termed the No-body, thus triggering their exosome-mediated degradation [142].

In addition to surveillance of aberrant pre-ribosomal assembly that occurs in the nucleolus, there might be quality control for mature ribosomes. Mature 40S and 60S ribosomes are monitored by nonfunctional rRNA decay (NDR) [143,144]. However, it appears that there are mechanistically distinct NDR pathways involved in turnover of 40S or 60S ribosomal subunits. In contrast to 40S subunits, it has been shown that nonfunctional 60S ribosomes are subjected to active ubiquitin-dependent degradation, which requires the ubiquitin protein ligase component Rtt101p and its associated protein Mms1p [143]. RP or other proteins associated with nonfunctional ribosome particles are ubiquitinated and degraded in an Rtt101p-Mms1p-dependent manner. Upon degradation of these proteins, naked nonfunctional rRNA is exposed to RNases and digested [143].

Finally, mature ribosomes are degraded by a specific type of autophagy, termed ribophagy, upon nutrient starvation [145]. This process requires the deubiquitination enzymes Bre5 and Ubp3 [145]. It remains



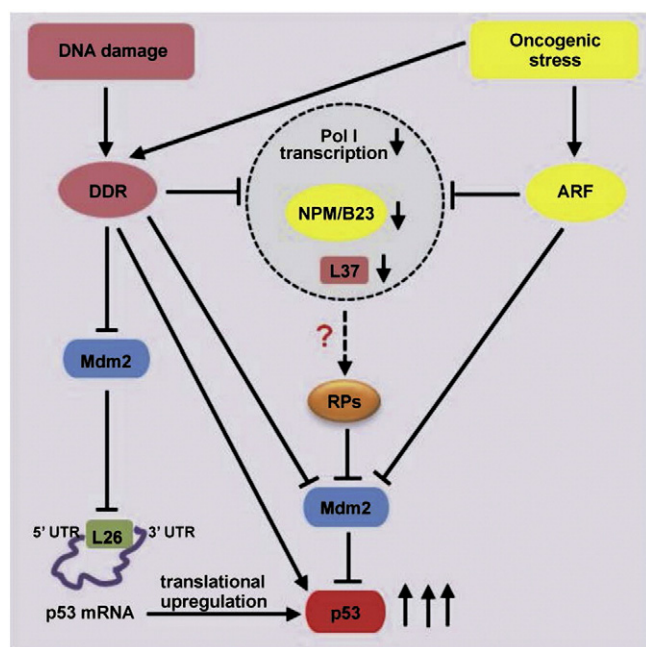
to be determined whether ribophagy also acts as a quality control mechanism able to remove unassembled or damaged ribosomes.

All these mechanisms were discovered in yeast, and similar mechanisms of ribosome biogenesis quality control operating in mammalian cells have yet to be uncovered. It will be of paramount importance to characterize the mechanisms by which a quality control system senses defects in ribosome biogenesis and to elucidate their relationship with the RP–Mdm2–p53 checkpoint signaling pathway. It can be hypothesized that the RP–Mdm2–p53 cell cycle checkpoint creates an extended time window to allow completion of removal of lesion in ribosome biogenesis prior cell division. Additionally, ribosome-free RPL11 and activated p53 could block various aspects of ribosome biogenesis by inhibiting c-Myc [50,131,133,134] or directly repressing Pol I and Pol III activities, import of RPs and export of ribosomal subunits respectively, until lesions in ribosome biogenesis are removed [61,146]. Given the role of p53 in the regulation of autophagy [147,148], it would be exciting to test the possibility that p53 regulates ribophagy upon ribosome biogenesis stress. There is abundant room for further progress in these areas of research.

## 6. Communication of the ribosome biogenesis stress signaling pathway with the DNA damage response, oncogenic signaling and splicing machinery

Although extensive evidence has been accumulated over the past decade that shows perturbation of ribosome biogenesis in the nucleolus can trigger p53 stabilization and activation independent of DNA damage and ARF via binding of several RPs to Mdm2 and inhibition of its E3 activity toward p53 [22,30,34], a number of studies strongly point to the fact that both DDR and ARF can inhibit ribosome biogenesis, and consequently engage several RPs in p53 activation (Fig. 4). Rubbi and Milner

first proposed the idea that most p53-inducing genotoxic stressors also inhibit ribosome biogenesis and compromise the structure of the nucleolus [37] (Fig. 4). They suggested that nucleolar disruption was required for induction of p53 in response to DNA damage, as induction of DNA damage without nucleolar disruption did not trigger p53 [37]. The repetitive nature of the rDNA region and the high rate of Pol I transcription, makes rDNA highly unstable. Thus, it has been proposed to act as a potential sensor for DNA damage [149]. UV-mediated DNA damage causes nucleolar disruption by directly inhibiting rDNA transcription as well as by global RNA Pol II transcription [37,89,150], whereas DSB lead to a transient repression of rDNA transcription via ATM–NBS1–MDC1-dependent interference with Pol I initiation complex assembly, which results in a premature displacement of elongating holoenzymes from rDNA [151]. But how do these nucleolar changes contribute to p53 activation upon DNA damage? It was originally suggested that impairments of nucleolar structure and function abrogate Mdm2-mediated p53 polyubiquitination in the nucleolus and its consequent proteasomal degradation, leading to p53 accumulation [37,152]. More evidence suggests that nucleolar disruption associated with DNA damaging stressors engages the aforementioned RP–Mdm2–p53 signaling pathway as well as other RP-dependent signaling pathways to boost the p53 response [22]. It was first shown that RPS7 and RPL11 are required for full p53 stabilization and activation in response to DNA damage [101,153]. Recently, Llanos and co-workers [57] uncovered an interesting mechanism by which RPL11 is engaged in p53 activation by DNA damage (Fig. 4). Treatment with cisplatin, UV light or doxorubicin decreased the level of RPL37, which caused ribosome biogenesis stress and consequently activation of the RPL11–Mdm2–p53 signaling pathway. Further research will be required to elucidate the underlying mechanism by which DNA damage leads to RPL37 degradation and determines whether this regulation is specific to RPL37 or is general to other RPs. In addition to RPL11 and RPS7, RPS3 was shown to trigger p53 stabilization in response to oxidative stress and associated DNA damage by interacting with both Mdm2 and p53 [99]. RPL26 was also shown to be a positive regulator of p53 at the translational level upon  $\gamma$ -irradiation (Fig. 4). Under these conditions RPL26 competes with the nucleolin homodimers for binding to both the 5'- and 3'-UTRs of p53 mRNA and consequently stimulates its translation [154–156]. However, the source of p53 mRNA-interacting RPL26 upon DNA damage remains to be determined. Under non-stressed conditions, Mdm2 inhibits RPL26 interaction with p53 mRNA as well as targeting it for degradation. In response to genotoxic stress, the inhibitory effect of Mdm2 on RPL26 is attenuated, enabling its binding to p53 mRNA and a rapid increase in p53 synthesis [97]. In addition to upregulation of p53 via its direct binding to Mdm2 [64,85], ARF can also suppress ribosome biogenesis at multiple levels upon oncogenic signaling (Fig. 4). First, ARF inhibits rRNA processing by binding to NPM1/B23 and mediating its degradation [157,158]. Second, it interferes with Pol I transcription by blocking UBF1 (upstream binding factor 1) phosphorylation or preventing the nucleolar import of TTF-1 (transcription termination factor 1) [159,160]. Third, ARF inhibits 5S rRNA synthesis by blocking Pol III transcriptional activity [161]. Consequently, ARF-mediated inhibition of ribosome biogenesis might activate the RP/5S rRNA–Mdm2–p53 pathway (Fig. 4). In support of this model, overexpression of ARF increased the levels of ribosome-free RPL11 [162]. In addition to this mechanism, oncogenic c-Myc can also trigger the RP–Mdm2–p53 pathway independent of ARF- or DNA damage-mediated ribosome biogenesis stress, probably by upregulating synthesis of RPs, which bind to Mdm2 and augment cellular p53 activity [30,61]. Recent overexpression experiments in cell culture showed that ARF and RPL11 cooperatively bind to Mdm2 and inhibit its ubiquitin protein ligase activity toward p53 [162]. Together, these observations suggest that the physical and functional interplay between the RPL5–RPL11–Mdm2–p53, ARF–Mdm2–p53 and DDR signaling pathways may provide an important anti-cancer barrier. However, the detailed molecular mechanisms whereby these signaling pathways communicate with each other



**Fig. 4.** Interplay between the DDR and ARF with the RP–Mdm2–p53 signaling pathway. Both DNA damage and oncogenes trigger the DDR. The stabilization and activation of p53 in response to DNA damage result from phosphorylations of p53 and Mdm2 that disrupt their interaction. Oncogenic stress also upregulates ARF, which binds to Mdm2 and inhibits its activities toward p53. Both the DDR and ARF inhibit Pol I transcription. Additionally, the DDR and ARF inhibit rRNA processing via degradation of RPL37 or NPM1/B23, respectively. The resulting ribosome biogenesis stress presumably triggers the RP–Mdm2–p53 signaling pathway (dashed arrow) and leads to the accumulation of ribosome-free RPL26. The inhibitory effect of Mdm2 on RPL26 is attenuated by the DDR, enabling its binding to p53 mRNA and its rapid translation. The relative contribution of the ribosome biogenesis stress signaling to p53 activation by the DDR and ARF remains to be elucidated.

upon various cellular stresses and their relative contribution to p53 activation and tumor suppression remain to be elucidated.

Splicing is a highly regulated step of gene expression. Alteration in this process can have deleterious consequences on the cell. It was recently shown that oncogenic serine/arginine-rich splicing factor 1 (SRSF1), which is overexpressed in many cancers, binds the RPL5–Mdm2 complex and stabilizes the tumor suppressor p53 [163]. This observation suggests that a p53-dependent ribosome biogenesis stress signaling pathway may also sense the infidelity of splicing to trigger p53-dependent cellular adaptation mechanisms, even though the authors did not clarify whether RPL5 is causative, or only permissive for this response [163]. It would also be important to determine the effect of RPL11 depletion in this model, given its essential role in p53 upregulation by the RP–Mdm2 checkpoint.

## 7. Role of aberrant upregulation of p53 in the pathogenesis of ribosomopathies

Based on concepts developed in the above-mentioned studies of mouse models of RP-insufficiencies, it was suggested that p53 could also be involved in mediating some of the phenotypic manifestations of human ribosomopathies, a diverse group of pathological conditions in which genetic abnormalities cause defective ribosome biogenesis and/or mature ribosome function [33]. Indeed, recent studies of mouse models of human ribosomopathies or patients with ribosomopathies point to a causative role of aberrant p53 upregulation in the pathogenesis of Treacher Collins syndrome (TCS), Diamond–Blackfan anemia (DBA), 5q-syndrome and possibly Shwachman–Diamond syndrome (SBDS).

### 7.1. Evidence from mouse models of ribosomopathies

TCS is an autosomal dominant disorder of human craniofacial development that results from loss-of-function mutations in the *Tcof1* gene [164], which encodes treacle protein that participates in rDNA transcription and rRNA modification during the early stages of processing [165,166]. The first strong indication that p53 plays a role in the pathogenesis of human ribosomopathies came from studies of *Tcof1*-heterozygous mice that accurately recapitulate human TCS [167]. Haploinsufficiency of *Tcof1* in these mice perturbs ribosome biogenesis, and consequently triggers p53-mediated apoptosis in neuroepithelial and neural crest cells [167]. Genetic and pharmacologic inhibition of p53 prevented apoptotic elimination of neural crest cells and fully rescued the craniofacial abnormalities in *Tcof1*-heterozygous mice [167], showing that aberrant p53 upregulation is the underlying cause of TCS.

Heterozygous mutations or deletions of RP genes have been implicated in the pathogenesis of two bone marrow failure syndromes in humans, DBA and the 5q-syndrome, a subtype of myelodysplastic syndrome (MDS) [33]. DBA is a congenital disease characterized by macrocytic anemia with decreased erythroid progenitors in the bone marrow. About half of affected individuals also have craniofacial and thumb malformations, anomalies of the heart and urogenital system as well as short stature. DBA patients have an increased risk of developing acute myeloid leukemia (AML) and solid malignancies [28]. In addition to *RPS19*, the first identified and the most frequent mutations in DBA [16], pathogenic mutations or deletions of other RP genes, including *RPL5*, *RPL11*, *RPS26*, *RPL35A*, *RPS7*, *RPS10*, *RPS17*, *RPL26*, *RPS24* and *RPL15* have been recently identified in about 50–60% of patients [168–180]. Interestingly, there are correlations between specific gene defects and the presence and type of congenital malformations. Mutations in *RPL5* and *RPL11* are associated with a higher overall frequency of occurrence of congenital malformations than in other DBA patients [169,171,176]. Furthermore, mutations in the *RPL5* gene are associated with a higher frequency of craniofacial abnormalities and mutations in *RPL11* with thumb malformations [169,171,176].

Deficiencies of any of mutated RP genes in DBA are associated with abnormal ribosome biogenesis within affected tissues of these patients, suggesting that a deficit of functional ribosomes is responsible for the pathological manifestations [15,181–184].

Several mouse and zebrafish models of DBA have been generated, which show overlapping and nonoverlapping phenotypes. Unfortunately, none of them fully recapitulate phenotypic features observed in DBA patients. These differences between these mouse models and DBA patients could reflect the influence of polymorphisms in genes discrete from the primary mutations or species-specific differences. Homozygous deletion of *Rps19*, the most commonly mutated gene in DBA, is lethal in mice at the early embryo stage [185]. However, heterozygous embryos develop normally, showing no feature of the human DBA syndrome later in life [185]. Subsequently, in a large scale mutagenesis screen in mice for pigmentation abnormalities, Dark skin 3 (*Dsk3*) mutant was isolated carrying heterozygous missense mutation (T316A) in *Rps19* [32]. Interestingly patients with DBA do not show pigmentation abnormalities [33]. As has been described in humans with DBA, *Rps19<sup>Dsk3</sup>* mice also displayed macrocytic anemia with decreased erythroid progenitors in the bone marrow and reduced body size. Phenotypic differences between these mice and the ones used by Matsson and colleagues [185] could be due to the different genetic background, or to a partial negative dominance of the mutated protein. Genetic inactivation of p53 in *Rps19<sup>Dsk3</sup>* mice rescued the erythrocyte and body weight phenotypes as well as the pigmentation phenotypes [32]. Hyperpigmentation was traced to p53-dependent upregulation of the Kit ligand in keratinocytes, which then stimulated melanocyte proliferation via a paracrine mechanism [32]. Consistent with this mouse DBA model, p53 was shown to mediate DBA-like hematopoietic defects in transgenic mice expressing shRNA against *Rps19* under the control of an inducible promoter [186]. The involvement of p53 in DBA was further supported by the rescue of various developmental abnormalities in *rps19*-deficient zebrafish upon suppression of p53 and its family member, deltaNp63 [187]. However, knockdown of p53 did not alleviate erythroid aplasia in *rps19*-deficient zebrafish, suggesting the existence of a p53-independent but *rps19*-dependent pathway that is responsible for defective erythropoiesis in this DBA model [188]. This differential role of p53 in the erythropoietic phenotypes of *Rps19*-deficient mice and zebrafish remains to be elucidated [32,188]. Recently, two mouse mutations of *Rps7* (*Rps7<sup>Mtu</sup>* and *Rps7<sup>Zma</sup>*), a gene that has been implicated in DBA, were found in an N-ethyl-N-nitrosourea mutagenesis screen [189]. In contrast to DBA patients, *Rps7<sup>Mtu/+</sup>* and *Rps7<sup>Zma/+</sup>* mutant mice do not display anemia [189]. They are characterized by reduced body size, abnormal skeletal morphology and mid-ventral white spotting, the phenotypes shared with some other RP-deficient mice [31,189]. Additionally, increased neuronal apoptosis leads to malformations of the developing central nervous system and defects in working memory, the phenotypes not seen in other RP-deficient mice or DBA patients [189]. Interestingly, they show a decreased viability, which tends to be highly variable, depending on genetic background. Inactivation of one *p53* allele largely rescued all morphological phenotypes of these mice and promoted their survival. Thus, p53 mediates both hematopoietic and non-hematopoietic phenotypes in these mouse and zebrafish DBA models, strongly suggesting that it may also play an important role in the pathogenesis of DBA [189].

The 5q-syndrome is caused by a somatically acquired deletion in the long arm of chromosome 5, and is characterized by macrocytic anemia with decreased erythroid progenitors in the bone marrow and an increased risk of developing AML, similar to DBA. Additionally, these patients present with thrombocytosis and megakaryocytic dysplasia [33]. On the basis of a small scale RNAi screen targeting 40 genes in the common deleted region (CDR) of the 5q-syndrome, *RPS14* was identified as a critical gene on 5q whose hemizygosity recapitulates erythroid abnormalities in 5q-MDS, a phenotype that was rescued *in vitro* by forced expression of *RPS14* in hematopoietic stem cells from the 5q-patients [190,191]. Recently a mouse model of the human 5q-



syndrome was generated by hematopoietic stem cell-specific Cre/loxP-mediated heterozygous deletion of a region of chromosome 18, which is syntenic for the *CD74–Nid67* interval of the CDR that contains eight genes, including *Rps14* [192]. These mice developed macrocytic anemia and monolobulated megakaryocytes, and their bone marrow progenitors show aberrant expression of p53 and signs of apoptosis. p53 deficiency in these mice ameliorated the macrocytic anemia, which is caused by *Rps14*-haploinsufficiency, suggesting an important role of the p53 responses in the pathogenesis of the 5q-syndrome [192]. Recently, a deficiency in the expression of two microRNAs that are transcribed from the CDR of the 5q-syndrome, miR-145 and miR-146a, has been shown in mice to contribute to thrombocytosis, megakaryocytic dysplasia and the increased risk of AML, but not the macrocytic anemia [193].

Shwachman–Diamond syndrome (SDS) is an autosomal recessive disorder characterized by exocrine pancreatic insufficiency, skeletal defects, chronic neutropenia and a propensity to develop MDS and AML [33]. Approximately 90% of SDS patients have been found to have bi-allelic mutations in the *SDBS* gene, whose product facilitates the release of eIF6, a factor that prevents joining of the 60S and 40S subunits into 80S ribosomes [194–196]. Knockdown of the zebrafish *sdb*s ortholog produces a phenotype resembling SDS patient pathology [197]. Whereas loss of p53 rescued the skeletal phenotype of *sdb*s-deficient zebrafish, it did not rescue the pancreatic defect and neutrophil deficiency [197]. Interestingly, knockdown of *sdb*s gene resulted in aberrant expression of genes involved in ribosome biogenesis in the pancreas, including *rpl3* and *pescadillo*. Knockdown or mutation of either *rpl3* or *pescadillo* recapitulated p53-independent effects of *sdb*s knockdown on the pancreatic development [197].

## 7.2. Evidence from patients with ribosomopathies

Consistent with studies on animal models of human disorders of defective ribosome biogenesis described above, mounting evidence suggests that activation of p53 may also underlie the pathogenesis of the human DBA, 5q-syndrome and Shwachman Diamond syndrome (SDS). The depletion of RPS14 or RPS19 by short harpin (sh) RNA in human CD34<sup>+</sup> hematopoietic progenitor cells led to induction of p53 and a p53-dependent cell cycle arrest and apoptosis in erythroid progenitor cells, suggesting a role for p53 in the pathogenesis of the 5q-MDS and DBA patients [58,198]. Importantly, accumulation of nuclear p53 was found in bone marrow biopsy samples from DBA, 5q-syndrome and SDS patients, suggesting the basis for the failure of erythropoiesis in these diseases [58,199,200].

It was surprising that p53 was also upregulated in primary hematopoietic progenitor cells from DBA patients with mutations in *RPL11* or the same cells from control subjects after the depletion of *RPL11* with shRNA, given that numerous cell culture experiments demonstrated that *RPL11* is a critical positive regulator of p53 upon impairment of ribosome biogenesis [19,22,94]. These results suggest that *RPL11* levels may be sufficient in these hematopoietic progenitor cells to trigger the p53 response [198]. Another possibility is that, in contrast to cell culture models [19,22,94], *RPL11*-deficiency may activate a p53-dependent checkpoint response, as previously observed in *rpl11*-deficient zebrafish [201]. Further studies of p53 in DBA, 5q-syndrome and SDS patients may provide insights into pathogenesis, not only of defective erythropoiesis, but also other developmental abnormalities and the cancer predisposition that are inherent to these diseases, as well as guide the development of new therapeutic approaches for ribosomopathies.

## 7.3. How does p53 contribute to tissue-specific pathological manifestations of ribosomopathies?

Mutations affecting components involved in ribosome biogenesis may result in decreased ribosome number or aberrant ribosomes, which can deregulate gene expression at the level of translation. Almost

40 years ago, Harvey Lodish postulated that the spectrum of translated mRNA varies with the overall number of ribosomes [202]. Messenger RNAs that have low affinity for translational machinery are out-competed with messages with high affinity when the number of ribosomes is reduced, whereas low and high-affinity mRNAs are translated when ribosome biogenesis is upregulated [203,204]. Thus, it is reasonable to assume that specific mutations that lead to a reduced number of functional ribosomes to mRNA transcripts in the cell would alter not only the rate of total protein synthesis, but also the patterns of translated mRNAs [205]. Furthermore, qualitative defects in ribosome biogenesis might affect the translation of specific target mRNAs [206–208]. Moreover, recent studies have suggested that some factors involved in ribosome biogenesis may participate in other important cellular functions [146,209]. However, the contribution of these processes to the pathological phenotypes of ribosomopathies will not be discussed here.

How can p53 be responsible for tissue-specific pathological manifestations in ribosomopathies? A possibility exists that tissue-specific up-regulation of p53 in response to inherited mutations in a component involved in ribosome biogenesis may be caused by tissue-specific defects in ribosome biogenesis. For example, the same mutation may allow normal ribosome production in tissues where rates of ribosome biogenesis, cell growth, and cell division are slow but not in highly proliferating tissues that require intense ribosome production [31,34]. Moreover, the extent by which mutation in a component involved in ribosome biogenesis will lead to inhibition of ribosome biogenesis, and consequently p53 upregulation, might also depend on the relative expression levels of that component in specific tissues, its requirement in ribosome biogenesis or the severity of the mutant allele [14,15,32,33,189]. Furthermore, other factors can determine whether or not this potential for rapid overproduction and accumulation of p53 in response to a deficiency in ribosome biogenesis can actually occur. Such factors could be expression levels of p53 mRNA or its upstream regulators in specific tissues or developmental stages as well as the genetic background of an affected individual [4,189,210].

p53 responses in specific tissues can depend on the expression levels of its target genes. For example, in response to a RP deficiency p53 down-regulates white spotting gene *Kit* in developing melanocytes, whereas in keratinocytes it upregulates the expression of *Kit* ligand (*KITL*), leading to skin hypopigmentation or hyperpigmentation, respectively [32].

Relatively little is known about p53 downstream targets in mouse models of ribosomopathies and in human patients. Gene expression profiling in *Rpl24*<sup>Bst/+</sup> mouse embryos and CD34<sup>+</sup> hematopoietic stem cells from patients with the 5q-syndrome identified a number of overlapping and non-overlapping p53 target genes that are significantly deregulated, including those involved in apoptosis (*FAS*, *TRAF1*, *CASP3*, *BAX*, *BID*, *cyclin G* and *DR5*), cell cycle regulation (*CDKN1a*), stress responses (*SES2*, *SES3* and *WIG1*), mTORC1 signaling (*SES2*), autophagy (*SES2*), DNA damage repair (*DDB2*), metastasis suppression (*CD82*) and p53 regulation (*MDMX* and *PARC*) [31,200]. However, further research is ultimately needed to identify additional p53 target genes that are deregulated upon impairment of ribosome biogenesis *in vivo* and understand the molecular links between these targets and tissue-specific phenotypes.

## 8. The role of the RPL5–RPL11–Mdm2–p53 signaling pathway in malignant transformation and tumor progression

It is well established that upregulation of translational capacity as a result of increased ribosome biogenesis contributes to cancer development and progression [211–213]. Recent evidence suggests that the RPL5–RPL11–Mdm2–p53 pathway may monitor excessive ribosome biogenesis to prevent tumorigenesis. One of the key regulators of ribosome biogenesis is c-Myc, which upregulates transcription of 47S rRNA, 5S rRNA, and all RPs [4,61,131,214]. In an attempt to assess the

importance of nascent ribosome biogenesis to the development of B-cell lymphomas overexpressing c-Myc under the control of the immunoglobulin heavy chain promoter and enhancer ( $\text{E}\mu\text{-Myc}$ ). Barna and co-workers crossed  $\text{E}\mu\text{-Myc}$  transgenic mice onto a *Rpl24*-heterozygous genetic background [215]. The onset of B-cell lymphoma in these mice was delayed. One explanation for this could be the re-normalization of protein synthetic rate as a result of inability of c-Myc to upregulate nascent ribosome biogenesis due to a limiting amount of Rpl24. However, given the fact that RPL24-deficiency triggers the RP-Mdm2-p53 signaling pathway [31], this mechanism might also be responsible for the delayed onset of B-cell lymphoma in these compound mice. Alternatively, the accumulation of free RPL11 upon *Rpl24*-haploinsufficiency may repress c-Myc's transcriptional activity by directly binding to it [134,135] or through a mir-24/miRISC-mediated mechanism [133–135]. The most convincing evidence yet that the RPL5-RPL11-Mdm2-p53 signaling pathway monitors the excessive ribosome biogenesis to inhibit tumorigenesis was provided recently by Macias and co-workers [30]. They showed that loss of the RPL5-RPL11-Mdm2 interaction in knock-in mice that express the mutant  $\text{Mdm2}^{\text{C305F}}$  significantly reduced the p53 levels and accelerated  $\text{E}\mu\text{-Myc}$ -induced B-cell lymphomagenesis. The results of this study also supported a model in which the RP5-RPL11-Mdm2-p53 and the ARF-Mdm2-p53 pathways are independent, parallel pathways working together to protect cells from  $\text{E}\mu\text{-Myc}$ -induced tumorigenesis [30]. Even though this is an important step forward in understanding the roles of RPL5 and RPL11 in tumorigenesis, we must be aware of certain limitations of this model. The  $\text{Mdm2}^{\text{C305F}}$  mutation may affect Mdm2's functions that are independent of RPL5-RPL11 binding, such as interactions with other regulatory or target partners. In contrast to  $\text{E}\mu\text{-Myc}$ -induced B-cell lymphomagenesis [30], the disruption of the RPL5-RPL11-Mdm2-p53 signaling by an  $\text{Mdm2}^{\text{C305F}}$  mutation did not accelerate prostatic tumorigenesis in a model in which the RB family members pRB/p107/p130 were inactivated [216]. These results suggest that RPL5-RPL11-mediated regulation of p53 is not essential for inhibition of tumorigenesis in this model. However, it remains to be determined whether inactivation of RB family members triggers the nucleolar stress response that results in accumulation of ribosome-free RPL5 and RPL11. Furthermore, the possibility exists that RPL5 and RPL11 may play a role in tumorigenesis independent of the Mdm2-p53 module.

The observations that patients with ribosomopathies have a higher incidence of malignancies and that heterozygous mutations of a number of RPs in *Drosophila* and zebrafish result in benign or malignant tumors strongly suggest that defective ribosome biogenesis may be an oncogenic event [28,217–219]. This is most likely related to quantitative or qualitative defects in mRNA translation, although mechanisms that are unrelated to protein synthesis can't be excluded. Since errors in ribosome biogenesis are frequently associated with activation of the RP-Mdm2-p53 signaling pathway, it can be speculated that induction of this response prevents expansion of such potentially hazardous cells. Studies in RP-deficient zebrafish provided some evidence in support of that speculation. Deficiencies of 17 individual RPs in zebrafish lead to the development of very rare malignant peripheral nerve sheath tumors (MPNST) [219]. It has been suggested that the lesion in translation in these zebrafish mutants acts as an initiation event for cancer [27]. Surprisingly, MPNST were frequently observed in zebrafish carrying inactivating mutations of p53 [220]. Subsequent work showed that RP-haploinsufficient zebrafish cells lose p53 expression at the level of translation [221]. Thus, one could argue that the selection of cells defective in a component that regulates translation of p53 mRNA in RP-deficient zebrafish could also play a role in their progression to MPNST.

An alternative explanation for tumorigenesis in these zebrafish models could be that decreased numbers of ribosomes impair selective translational upregulation of mRNAs encoding for the key tumor suppressor p53. Consistent with this suggestion, selective defects in the translational upregulation of IRES-containing mRNAs, including p53

mRNA, were observed in patients with X-linked dyskeratosis congenita (DKC), the rare multisystemic syndrome which is caused by mutations of the *DKC1* gene encoding dyskerin, a highly conserved pseudouridine synthase that catalyzes pseudouridylation of rRNA [206,222]. This defect in translational upregulation of IRES-containing mRNAs was traced to a decreased pseudouridylation of rRNA, which affects the affinity of the ribosome for these mRNAs [206,223], and it was suggested to be responsible for an enhanced susceptibility to cancer in these patients [206,223]. The idea that p53 acts as a major barrier to tumorigenesis caused by impairment of ribosome biogenesis was also supported by studies of the 5q-syndrome in humans. Nuclear p53 accumulates in erythroid progenitor cells from these patients [58,200] and it is responsible for the failure of erythropoiesis [192]. By using sensitive deep-sequencing technology, it was recently demonstrated that *TP53* mutations occur in almost a fifth of 5q-MDS patients. Importantly, these mutations were associated with an increased risk of developing acute myeloid leukemia [224]. Taken together, the selection of cells that lost expression of wild-type p53 or acquired mutations in p53 or other signaling components of the RP-Mdm2-p53 signaling pathway may allow the pre-neoplastic cells to escape from the ribosome biogenesis stress-imposed checkpoint, thereby facilitating malignant transformation and tumor progression. These cells could also be unresponsive to some other exogenous or endogenous stresses that upregulate p53, putting them at an even greater risk of malignancy [23,24]. In addition to abrogating the tumor suppressor functions of wild-type p53, p53 mutations may also endow the mutant protein with new activities that can contribute to various stages of transformation and tumor progression as well as to increased resistance to anticancer treatments [225].

Although accumulating evidence suggests that RPL5 and RPL11 may play a role in tumor suppression by upregulating the p53 responses and inhibiting c-Myc function, no direct *in vivo* proof has yet been reported to verify RPL5 and RPL11 as bona fide tumor suppressors [22,133–135]. It might be predicted that mutations in *RPL5* and *RPL11* could have arisen in cancer cells to allow them to escape potential tumor suppressive functions of these RPs. Identification of cancer-associated *RPL5* and *RPL11* mutations and characterization of their functional significance *in vitro* and *in vivo* will be needed to demonstrate conclusively that RPL5 and RPL11 are bona fide tumor suppressors. The possibility exists that *in vivo* effects of these mutations on tumorigenesis may not be the equivalent to the  $\text{Mdm2}^{\text{C305F}}$  mutation [30], as RPL5 and RPL11 may have Mdm2-independent functions [133–135].

## 9. Conclusion

Intense research efforts over the last decade have revealed the existence of a p53-activating checkpoint signaling pathway triggered by impaired ribosome biogenesis with implications for the pathogenesis of human diseases [19,22,33,94]. The importance of RPL5, RPL11 and 5S rRNA in the regulation of the Mdm2-MdmX-p53 module upon impairments of ribosome biogenesis appears well established. On the other hand, additional studies will be necessary to unambiguously ascribe “p53-activating” functions to other RPs previously shown to bind Mdm2 under these conditions. A systematic approach will be required to identify additional components of this novel checkpoint signaling pathway, including those involved in sensing the lesion in ribosome biogenesis, transmitting the signals to the Mdm2-MdmX-p53 module and integrating it with signaling pathways that regulate other biological processes. Since many genotoxic and non-genotoxic stressors compromise ribosome biogenesis and nucleolar structure, it will be important to gain insights into the functional interplay between ribosome biogenesis stress checkpoint signaling pathway with other stress signaling pathways, such as the DDR and oncogenic signaling [37]. Classification of the molecular functions of nucleolar proteins suggests that only 30% participate in ribosome biogenesis. The other functions include regulation of telomerase function, senescence, aging, biogenesis of multiple RNPs, apoptosis, viral infection, DNA replication, and repair [149]. It

could be speculated that impairments of ribosome biogenesis may also contribute to the pathogenesis of ribosomopathies via alteration of these functions. It also remains to be determined whether the molecular mechanisms that lead to degradation of the aberrant pre-ribosomal assemblies are an integral part of the ribosome biogenesis stress checkpoint. The most challenging questions in the field are related to the genetic manipulation of ribosome biogenesis stress checkpoint genes in mice and other model organisms. It will be of paramount importance to elucidate whether normal functions of the ribosome biogenesis stress checkpoint are compromised in various human diseases. The results of the aforementioned studies will contribute to a better understanding of the pathogenesis of a number of human diseases, including ribosomopathies and cancer, as well as guiding the development of new therapeutic approaches for these diseases.

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